Sulphur Distribution in Fish Flesh Proteins

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ABSTRACT

The sulphur distributions in the crude flesh proteins of lingcod, halibut, lemon sole, and white spring salmon have been determined. The proteins of the species examined proved to be excellent sources of methionine and the figures obtained for each amino acid agreed closely amongst the different species.

Many and diverse properties of proteins in nutrition have been brought to light in recent years. Investigators have demonstrated injurious effects of protein deficiency on the healing of wounds, resistance to infections, chloroform liver injury, etc. (cf. Elman 1944, Stare, Hegsted and McKibbin 1945, Co Tui 1946, and Stone and Davidson et al. 1945). These properties are in the main a function of the constituent essential amino acids. On the other hand Woolley's (1946) report, in which he describes the existence of a peptide or peptide-like substance associated with proteins which accelérates growth, indicates that in so far as this function is concerned, amino acids of the protein may not be wholly responsible for the effects observed. This factor has been termed strepogenin; at time of writing, however, Woolley's work is difficult to assess because of the meagre information given in his reports. For example, in describing the effect of the newly discovered substance on the growth rate of mice, he neglects to report food intakes. The effect observed may, therefore, merely be one of palatability. Until further evidence is forthcoming it may be assumed that the nutritional properties of proteins are in the main a function of the essential amino acids contained therein.

It is, therefore, highly important that data be obtained by the best available methods on the important protein foods. The present report contains the values derived for methionine and cystine in lingcod (*Ophiodon elongatus*), halibut (*Hippoglossus stenolepis*), lemon sole (*Parophrys vetulus*), and white spring salmon (*Oncorhynchus tschawytscha*). Recent publications describing certain interesting physiological properties of these substances such as their effect on liver fat levels (Beeston and Channon 1936, Tucker and Eckstein 1937), their ability to prevent chloroform injury in hypoproteinemic dogs (Miller, Ross and Whipple 1940) and the transfer of the essential methyl group of methionine to form choline *in vivo* (Du Vigneaud *et al.* 1940) emphasize the desirability of acquiring analytical data on these amino acids in proteins of nutritional importance.

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EXPERIMENTAL

Samples of the fish flesh separations used in determining their biological values as described by Beveridge (1947) were utilized for the analyses presented in this paper. Fat was extracted by allowing 300 g. of meal to soak for 48 hours in 3000 cc. 3:1 alcohol-ether solution. The materials were then filtered off and extracted with ether in a soxhlet apparatus for about 24 hours. The air-dried substances were extracted with three successive 3000 cc. portions of boiling water. The lingcod and lemon sole preparations could not be filtered efficiently in this

TABLE I. Proximate analyses of crude fish flesh proteins. Nitrogen and sulphur figures are based on ash- and moisture-free material.

Source of protein	Moisture (%)	Ash (%)	Nitrogen (%)	Sulphur (%)
Lingcod	3.21	1.31	16.51	1.213
Halibut	2.29	0.71	16.50	1.183
Lemon Sole	3.68	1.20	16.62	1.177
White spring salmon	2.95	0.67	16.60	1.166

TABLE II. Sulphur distribution of fish flesh proteins. In no case was inorganic sulphur present in determinable amount.

Source of protein	Methionine (%)		Cystine (%)		Sulphur by summation (%)	Sulphur by oxidation (%)	Recovery (%)
	Author	Others	Author	Others			
Lingcod	3.82		1.46		1.211	1.213	99.8
Halibut	3.66		1.47	1.45*	1.179	1.183	99.7
Lemon sole	3.72		1.40		1.173	1.177	99.7
White spring salmon	3.64	3.78**	1.36	1.27*	1.146	1.166	98.3

*Pottinger et al. 1939.
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procedure and were subjected to centrifugation to get rid of the hot water. The meals were dried in a vacuum oven at 40 to 45° C. and placed in stoppered bottles.

In the proximate analyses of crude fish flesh proteins (table I), the moisture of the protein preparations was estimated by drying samples to constant weight *in vacuo* over phosphorus pentoxide; sulphur was determined as sulphate after fusion with sodium peroxide and sodium carbonate. Nitrogen was determined by the macro-kjeldahl method.

For the sulphur distribution (table II), analysis was carried out in triplicate or quadruplicate by the Kassell and Brand modification (1938) of Baernstein's method (1936a). The use of a mercury seal between the digestion flask and

reflux condenser, as recommended by the former workers, was found to be unnecessary. Although the data reported from the literature were obtained by different methods, the agreement is good. Representative values for methionine and cystine in casein, beef muscle and egg albumin obtained by the same methods as utilized by the author are given in table III.

An examination of tables II and III reveals that egg albumin contains definitely more methionine and slightly more cystine than any of the proteins cited. This material probably has the highest methionine content of any of the common protein foods. In their excellent monograph on the amino acid composition of

TABLE III. Methionine and cystine contents of casein, beef muscle and egg albumin.

Protein	Methionine (%)	Cystine (%)	Reference
Casein (Labco).....	3.15	0.53	(1)
Casein (Labco).....	3.17	0.39	Kassell and Brand 1938.
Casein (Harris).....	3.31	0.29	Baernstein 1936 a, b.
Beef muscle.....	3.19	0.97	(1)
Egg albumin.....	5.07	—	Baernstein 1936 a.
Egg albumin.....	5.23	1.78	Kassell and Brand 1938.

(1) These values were reported in a paper by Beveridge, Lucas and O'Grady (1945) uncorrected for moisture and ash content. They are presented here on a corrected basis.

proteins and foods, Block and Bolling (1945) list no other protein with as high a content of methionine as egg albumin. As a class, fish flesh proteins appear to be the next best source of this essential amino acid. The figures herein reported are higher than those obtained in the same way on casein and beef muscle. This finding is probably partly responsible for the relatively high biological values obtained with fish muscle proteins (cf. Beveridge 1946) for it is well known that the sulphur-containing amino acids are the main limiting factors in casein for the promotion of growth (Mulford and Griffith 1942).

SUMMARY

The sulphur distributions of the crude flesh proteins of lingcod, halibut, lemon sole, and white spring salmon have been determined.

Within the limits of experimental error all the sulphur present was accounted for in the form of methionine and cystine, thus precluding the existence of appreciable quantities of other sulphur-containing compounds.

The methionine and cystine values found in the different species varied from 3.64 to 3.82% and 1.36 to 1.47% respectively.

These figures indicate that of the proteins important in human nutrition, except for egg albumin, the crude proteins of fish constitute the best source of methionine.

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Silver Electrodes for Sterilizing Sea-water

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ABSTRACT

From experimental evidence and theoretical consideration there is nothing to indicate that sea-water or saline solutions can be sterilized by means of silver ions produced by electricity from silver electrodes.

Sea-water is usually preferred to fresh-water by fish processors for washing marine fish. There are two reasons for this: fresh water tends to precipitate the globulins on cut surfaces, giving the fish a less attractive finish; and, perhaps more important, sea-water is available in unlimited quantities, whereas fresh water frequently has to be purchased from some near-by municipal supply.

It sometimes happens that a fish plant obtains its water supply from a harbour that is continually receiving an inflow of raw sewage. The result is that the water supply is polluted and should not be used on fish or any other food without treatment.

Table I. Concentration of silver ion required to destroy certain non-spore forming bacteria suspended in water, as reported by various authors. Time of exposure, where reported, was between 1 and 3 hours.

Minimum lethal concentration (g. per cc.)	Test organism	Author
10^{-7} to 5×10^{-7}	*E. coli*	Tracy (1941)
3.5×10^{-7} to 4×10^{-7}	*E. coli*, etc.	Hoffman (1938)
2×10^{-7} to 4×10^{-7}	*E. coli*	Goetz (1943)
5×10^{-8}	*E. coli*	Hermann (1935)
7.5×10^{-8}	———	Dimitriev (1935)
2×10^{-7}	*E. coli*, etc.	Metalnikov (1935)
5×10^{-7} to 6×10^{-6}	———	Brandes (1934)
10^{-7} to 1.5×10^{-7}	*E. coli*, etc.	Myers and Mauer (1935)

The search for an economical and efficient method for the purification of sea-water led us to investigate the well-known bactericidal action of minute traces of silver ions (table I) to determine its efficiency in the presence of sodium chloride and other salts normally present in sea-water.

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Two investigations were carried out: (1) with commercial water-purifying equipment which utilized the principle of electrically produced oligodynamic silver as a means of destroying bacteria; (2) by using silver ions produced from solutions of silver salts such as silver nitrate.

The commercial equipment consisted of three separate mechanisms combined into one unit: (1) Silver ions generated by means of metallic silver electrodes, using a direct current, six volts, and from 0 to 1000 milliamperes as required or as the conductivity of the water permitted; (2) aluminum electrodes producing a hydrated aluminum oxide flock; (3) a sand filter about 30 inches deep. The

TABLE II. Reduction of bacteria in polluted sea-water treated with silver and aluminum electrodes and filtered through sand.

Plate counts at 25°C.			*E. coli* per ml.#	
Raw	Treated	Per cent efficiency	Raw	Treated
33000	25000	24.3	100	100
6200	7700	0	100	100
1200	500	58.3	100	100
12000	600	95.0	100	10
6900	110	99.8	100	1
5600	900	83.9	100	1
2100	570	72.8	100	1
20400	9000	55.8	100	100
2300	120	94.7	100	10
2900	113	96.0	1000	100
5700	107	98.1	100	10
8400	211	97.4	1000	100
1440	45	99.6	100	10
1670	160	90.4	100	100
1480	760	48.6	1000	10
3400	300	98.8	1000	100
2700	102,000*	0	100	10,000*

*—5 minutes after backwashing.
#—test made only on serial dilutions of from 1 to 10^{-5}.

reason for using this particular equipment was that it was readily available and is typical of the best of the modern apparatus using ionic silver as a means of purifying water.

ACTION OF COMMERCIAL PURIFIER

The commercial purifier was run continuously for about three months. During this time tests were made to determine its efficiency as a unit on the bacteria in the water passing through it. Bacterial analyses were made on the water before and after treatment. Plate counts were made on standard nutrient agar incubated

at 25°C. and 37°C. Counts were also made at 25°C. with Zobell's medium for growing marine types of bacteria. Serial dilutions down to 1 in 10,000 were tested for the presence of *E. coli* using standard methods of water analysis. The reduction in bacterial count (table II for typical results) varied from 0 to 99.3 per cent, with many counts showing a reduction above 95 per cent. *Escherichia coli* was never completely eliminated. The plate counts at 37°C. and those on Zobell's medium showed reductions similar to those indicated in the table. Examination of the colonies on the plate made from the treated water did not show a high proportion of spore-forming types.

In these tests the reduction indicated may have been the result of either the filter or the silver ions, or a combination of the two.

Attempts to measure silver in the treated sea-water using p-dimethylaminobenzal-rhodanine (Feigl 1928), dithizone (Fischer 1929 and 1930), or by the catalytic reduction of manganese and cerium salts (Feigl and Fränkel 1932), were unsuccessful. Either the silver was present in amounts too small to be measured by these methods, or the results were inaccurate because of the interfering action of other ions in the sea-water.

Baylis (1930) reported that with somewhat turbid water, by using an aluminum hydroxide coagulant and a sand filter, he was able to obtain a reduction of 95 per cent in the number of bacteria in water. Kempf and his co-workers (1942) removed 99.6 per cent with a similar type of filter. There is nothing, therefore, in the results recorded above that might not be obtained by the use of the coagulating electrode and the sand filter without the action of the silver electrode. To determine more specifically what action, if any, the silver electrode had on the bacteria, a further series of tests were run in the laboratory. In this case the flocculent and the sand filter were omitted and the silver was used alone.

ACTION OF SILVER ELECTRODES

Two 20-litre carboys were filled with tap water and sufficient sodium chloride was added to one to give a concentration of 2.5 per cent. Both were inoculated with a heavy suspension of *E. coli*. These waters were then run through a small purifier containing a silver electrode at the rate of approximately 2 l. per min., and samples of the treated water were collected in sterile glass containers. They were then plated out immediately following the treatment and then periodically during the following 60 minutes. The current passing through the silver electrode with the saline solution was approximately 100 milliamperes; with the tap water it was too small to be read accurately, but was in the neighbourhood of 0.7 milliamperes.

In the plotted results of the bacterial counts (fig. 1), it can be seen that in the tap water the silver acts as a typical disinfectant, giving almost a straight line reduction, characteristic of monomolecular reactions. With the saline solution, instead of a decrease the count had doubled at the end of an hour.

This experiment was repeated with the current passing through the saline solution increased from 100 to between 450 and 470 milliamperes. The results

were approximately the same. In addition to those with *Escherichia coli*, other tests were made with *Proteus vulgaris* and again the results were the same.

Polluted harbour water with its normal microflora and also with added inoculations of various cultures gave results similar to those with 2.5 per cent saline solution. After standing 8 to 10 hours subsequent to treatment with silver the number of organisms increased from 12 to 20 times.

In one of the large commercial purifiers four silver electrodes were inserted at a point after the water had passed through the sand filter. If this final silver treatment had any germicidal action, the count in the water should continue to

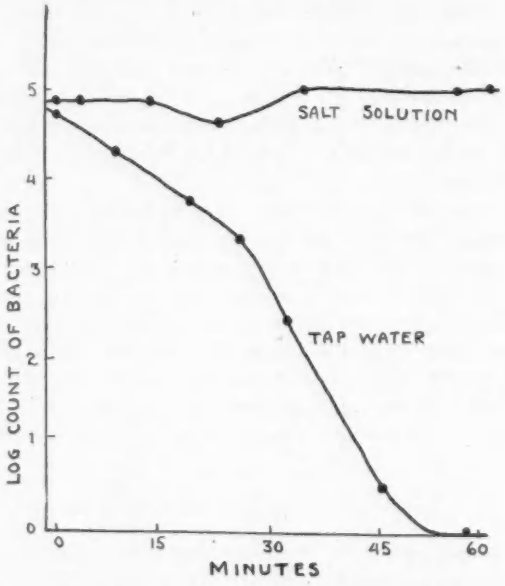

FIGURE 1. Changes in numbers of bacteria in tap water and in 2.5 per cent salt solution, each inoculated with a heavy suspension of *E. coli* and then passed through the silver sterilizer.

decrease on standing. However, successive counts on several treated samples showed no decrease over four-hour periods.

These tests all indicate that sodium chloride interferes with the bactericidal action of the silver electrode on bacteria in water.

EFFECT OF CHLORIDE ON CONCENTRATION OF SILVER IONS

It is known that the toxic action of silver is the result of the silver ions present in solution. Myers and Mauer (1935) have compared the germicidal activity of five forms of silver in sterilizing water. The results they obtained with silver chloride, silver nitrate, silver acetate, colloidal silver and a silver solution prepared by the Katodyn process, showed that, regardless of the nature of the com-

pound, solutions yielding similar concentrations of silver ions had equal germicidal value.

It seemed obvious, therefore, that a confirmation of the results obtained in the preceding group of experiments might be obtained by substituting solutions of silver ions in place of the silver electrode.

Three sets of nine 500-ml. flasks were prepared containing serial dilutions of silver nitrate in water. The concentrations of silver ions ran from 10^{-1} to 10^{-9} g. per ml. One set was made with distilled water, the second was made with 2.5 per cent saline solution and the third with sea-water.

These flasks were each inoculated with 1 ml. of a suspension of *E. coli*. Ten minutes after inoculation they were plated out on standard nutrient agar and the counts were made after 72 hours' incubation at 37°C.

Although the same number of silver ions were added to the fresh water, saline solution and sea-water, they will not remain so. As will be shown later in the discussion, because of the relative insolubility of silver chloride, the union of Ag^+ and Cl^- will remove all but slight traces of silver ions from the solution. No matter how much silver chloride is present, the concentration of silver or chloride ions should remain always the same.

The results of these tests (table III), show that with the lower concentrations of silver the sodium chloride did interfere with its toxic action. It is of interest to note that with the concentration of silver ion of 10^{-5} grams per ml. or more, the salt apparently did not interfere with the action of the silver. In both cases the silver ion concentration should be the same.

TABLE III. The effect of various concentrations of silver ions on the survival of bacteria when added to suspensions of organisms in distilled water, 2.5 per cent saline solution and sea-water with a 10-minute contact period.

Added silver ions (g. per ml.)	Bacterial count per ml.					
	Distilled water		Saline solution		Sea-water	
10^{-4}	0*	0	0	0	0	0
10^{-5}	0	0	0	0	87	119
10^{-6}	0	0	30	72	++	++
10^{-7}	0	0	++	++	++	++
10^{-8}	4	8	++	++	++	++
10^{-9}	560	610	++	++	++	++
Controls	3700	4100	5200	3800	3900	4300

*Results from duplicate counts.

++ = too many colonies present to be counted on the plates poured from the 1 or 0.1 ml. dilutions.

In repeating this experiment an observation was made that threw some light on this point. It was noted that while no growth occurred in plates poured from the first (1 ml.) or second (0.1 ml.) dilutions from some of these samples, colonies very slowly developed from the third (0.01 ml.) dilutions. In others, colonies developed from the second and third but not on plates from the first dilution. This would indicate that silver chloride beyond a certain concentration may have

a bacteriostatic action, and that the apparent destruction of bacteria in these plates is caused by their inability to grow and not that they have been killed. It is also possible that in some of the plates silver chloride may have a slow toxic action. Although the initial concentration of silver ions may be insufficient to destroy the organisms immediately, the silver chloride may act as a reservoir replacing silver ions as they are taken up by the bacteria and ultimately providing a sufficient amount to be lethal.

DISCUSSION

Since the time of Nägeli (1893) it has been known that traces of silver and other heavy metals have a toxic action on bacteria. Neisser and Eichbaum (1932) were the first to show that the toxic action was caused only by silver ions, and this has been confirmed by many others (Sollmann 1932, Gibbard 1933, Myers and Mauer 1935, etc.). Therefore anything that tends to remove silver ions from a solution will remove its germicidal activity.

The solubility of silver chloride is low: 0.000015 grams per 100 ml. water at 20°C. (Handbook of chemistry and physics 1945). At 25°C. the maximum number of silver ions in a 2.46 per cent by weight saline solution (2.5 per cent w/v) is therefore of a very low order:

$$Ksp = [Ag^+]\,[Cl^-] = 1.56 \times 10^{-10}$$

$$[Ag^+] = \frac{1.56 \times 10^{-10}}{[Cl^-]} = \frac{1.56 \times 10^{-10}}{\left(\frac{2.46}{5.84}\right)} = 3.70 \times 10^{-10}\ N_1,$$

which is approximately 4×10^{-11} grams per ml. This agrees well with the theoretical concentration of silver ions based on calculations using experimentally obtained activity coefficients (Glasstone 1942) for sodium chloride solutions, which is 0.4×10^{-10}.

It has been shown by tests made at this Station (table II) and in the records of tests made by others (table I) that 3×10^{-11} grams of silver ions per ml. is below the point where silver has any significant toxic action on bacteria. Furthermore, in sea-water, as well as chlorides there are traces of other substances which may still further reduce the concentration of silver ions.

With a decrease in temperature, the solubility of silver chloride decreases, and as sea-water during many months of the year is between 0° and 10° C., there is even less reason to expect any significant bactericidal activity.

Some confirmation of these facts is found in the work of others in closely related fields. Moiseev (1937) in sterilizing water by passing it through silver coated sand found that the presence of sodium chloride interfered with the process. Laurell (1933) pointed out that the toxicity of certain waters procured from metal stills is due to traces of the metallic ions (silver ions) in solution and showed that the use of physiological saline solution neutralized this toxic action, and Herrmann (1935) states that sodium chloride inhibits the effect of Katodyn silver in sterilizing water.

A contrary opinion is expressed by Zobell (1946, pp. 31-32). In speaking of the bactericidal action of metal containers used for collecting water samples, he lists copper, lead, nickel, silver, tin and zinc as being oligodynamic, and then states that the effect of these metals is greater in sea-water than in fresh water. It may be that this generalization is based chiefly on experience, using brass, copper, zinc, nickel or other metals whose chlorides are not only toxic but much more soluble than that of silver.

There is nothing, therefore, in either the experimental evidence or from theoretical considerations to indicate that silver ions will be produced in sufficient quantities in sea-water from silver electrodes to have any effect on the reduction of bacteria.

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Growth of *Clostridium* in Seaweeds and Marine Fish

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ABSTRACT

Some common seaweeds from the coastal waters of Nova Scotia supported the growth of certain saccharolytic species of *Clostridium*, especially *Cl. pasteurianum*. They did not support the growth of proteolytic species.

Under anaerobic conditions many proteolytic species of *Clostridium* grow in cod, hake, pollock and lobster from small inocula. Saccharolytic types grow poorly in fish and require large inocula to initiate growth.

INTRODUCTION

Relatively little is known of the part played by species of the genus *Clostridium* in the spoilage of sea fish and other marine products. In fact, apart from being found occasionally in canned products, the literature on fish spoilage is significantly devoid of any mention of species of this genus. In their investigations of the bacteria responsible for fish decomposition no mention is made of these obligate anaerobes in the papers of Gee (1927, 1930), Harrison (1929), Sanborn (1930), Gibbons (1933), Watson (1939), or Wood (1940). Chertkova *et al.* (1942) studying the successive changes in the microflora of frozen fish held in storage, observed the presence of *Clostridium* only after prolonged storage, and after spoilage had already occurred through the activity of gram negative rods.

Numerous studies have been made of the microflora of fish faeces, fish slime, and other materials considered to be the source of bacteria contaminating commercial fish. Reed and Spence (1929) and Shewan (1938) found *Clostridium* present in the intestinal contents of haddock. Snow and Beard (1939) observed none in the faeces of North Pacific salmon, and Kiser and Beckwith (1944) found none in the faeces of mackerel. However, as pointed out by Griffiths (1937), and Wood (1940), fish have no specific intestinal flora, but rather that of the environment in which they are feeding.

Observations on the microflora of fish slime invariably show it to be free from obligate anaerobes (Reed and Spence 1929, Stewart 1932, Snow and Beard 1939).

In a recent survey of the bacterial flora of normal fish, Thjøtta and Sømme (1943) isolated and classified organisms from the skin, gills, stomach and intestines of 15 sea fish and 4 fresh water species. No strict anaerobes were found.

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The seeming absence of *Clostridium* from the microflora of spoiling fresh fish may be explained by one of two hypotheses, or a combination of both: (1) the environment supplying the bacteria contaminating the fish may be so free from *Clostridium* that their growth rarely or never occurs; (2) the physical or chemical characteristics of the fish itself or the conditions under which it is handled may not be conducive to the growth of these obligate anaerobes.

If it becomes apparent that in marine products these organisms are absent, or have no significance in their decomposition, some explanation may be found for the reason why fish differ in this way from meats and vegetation of land origin.

With the object of gaining some more definite information on this subject, a study is being made of the growth of *Clostridium* in fish and other marine products. The results will be published in a series of papers, of which this is the first.

Source and Purity of Cultures

The cultures used in the tests outlined in these papers are from three sources. *Cl. botulinum*, types A, B, and C, and one culture of *Cl. butyricum* are from the American type culture collection. Those designated as being from spoiled canned fish, fish faeces, ocean mud, etc., were isolated and classified in this laboratory. The remainder were obtained through the courtesy of Dr. G. B. Reed of Queen's University.

Before use, the cultures were plated out and fresh strains re-isolated from single colonies. Sufficient tests were then made to insure the correct identity of each culture.

GROWTH IN SEAWEEDS

The natural habitat of the genus *Clostridium* is in the humus-rich layers of the soil. They grow abundantly in plant and animal tissue of land origin. Whether or not these organisms can grow in seaweeds may have an important influence on their numbers and distribution in the ocean, and this in turn on the initial contamination of fish products.

Portions of seven common seaweeds, *Chorda flagelliformis*, *Chorda filum*, *Laminaria digitata*, *Laminaria agardhii*, *Laminaria longicruris*, *Ascophyllum nodosum* and *Fucus vesiculosus*, gathered along the Nova Scotia coast, were desiccated and powdered in the Station's fish drying tunnel and a Wiley mill. These were prepared for growing bacteria by suspending five per cent in water and autoclaving in deep tubes. Both distilled water and seawater were used and some sets were buffered with an excess of $CaCO_3$. Also in some tests 0.1 per cent agar was added to provide better anaerobic conditions. Other portions of the seaweed were cut into pieces small enough to fit into test tubes and then autoclaved without the addition of other materials. The pH of the unbuffered autoclaved seaweeds ranged from 5.7 to 6.6.

The seaweed materials were inoculated with one ml. of dilutions made from pure cultures of the species. For comparison similar inoculations were made into either Reed's modification of Brewer's medium (Reed and Orr 1941) corn-liver mash (McClung and McCoy 1934) or a medium prepared from dried grasses (Castell 1942a).

The results of these tests were very definite, as is shown in a typical set of results given in table I. The proteolytic and "stinker" species as well as certain non-proteolytic species did not grow in any of the seaweeds, even though the inocula contained up to 10^{-7} or 10^{-8} viable cells, as shown by growth in other media. The following species are those which failed to grow in the seaweed: *welchii, sporogenes, paraputrificum, septicum, putrificum, botulinum and tertium.*

Cl. pasteurianum, Cl. butyricum, Cl. difficile, Cl. bifermentans and *Cl. multifermentans* grew in most of the seaweed media made from low dilutions. That is, a heavy inoculation was required to initiate growth. From a thousand to a million times as many cells were required to initiate growth in the seaweeds as were required in grass or corn-liver media, and of these *Cl. pasteurianum* consistently grew from higher dilutions than any of the other cultures.

TABLE I. The highest dilutions initiating growth of *Clostridium* in various seaweeds and in grass, corn-liver mash and in Reed's modification of Brewer's medium. Tests made in triplicate; figures given indicate that at least two of the three tubes were positive. 0 = no growth in any dilutions of 10^{-1} or higher.

Medium	*Cl. putrificum*	*Cl. botulinum* (type A)	*Cl. sporogenes*	*Cl. butyricum*	*Cl. past.*
Brewer's medium	10^{-8}	10^{-7}	10^{-8}	10^{-5}	10^{-7}
Corn-liver mash	10^{-6}	10^{-3}	10^{-7}	10^{-7}	10^{-4}
Grass medium	10^{-6}	10^{-1}	10^{-9}	10^{-7}	10^{-8}
Ch. filum	0	0	0	10^{-1}	10^{-4}
Ch. flagelliformis	0	0	0	0	10^{-1}
Lam. digitata	0	0	0	10^{-1}	10^{-2}
Lam. agardhii	0	0	0	10^{-2}	10^{-3}
Lam. longicruris	0	0	0	10^{-4}	10^{-5}

SUMMARY AND DISCUSSION

These results show that the algae used in these experiments are unable to support the growth of the putrefactive and "stinker" species, even when the inoculum contains one hundred million viable cells per ml. The saccharolytic species are able to utilize the seaweeds as a substrate for growth. They require a larger inoculum in the seaweeds than is required to initiate growth in tissue of many green plants of land origin or standard anaerobic media. Of all the organisms tested, *Cl. pasteurianum*, which is able to fix atmospheric nitrogen, grew best in the seaweeds.

These results are not unexpected. Waksman and Carey (1933) and Waksman, Carey and Reuszer (1933) have shown that similar types of algae are not readily decomposed by bacteria in seawater, because of their very low nitrogen content. With the addition of ammonia or nitrate salts, or sea bottom mud which supplies sufficient nitrogen, the algae were readily decomposed by marine bacteria.

The proteolytic species grow best in media rich in organic nitrogen and it is not surprising that they do not grow in a substrate with insufficient nitrogen

to support the ordinary mixed microflora of the sea. On the other hand the saccharolytic species, and especially *Cl. pasteurianum*, can grow in media rich in carbohydrates and relatively poor in proteins and other sources of nitrogen.

It is interesting to observe that Waksman and his co-workers found nitrogen-fixing species in marine sediments. Beneke and Keutner (quoted by Waksman, Hotchkiss and Carey 1933) were able to demonstrate that *Cl. pasteurianum* was found in the lower layers of the sea and in sea bottom sediments.

When these results are compared with the growth of the same organisms in a wide variety of fruits, vegetables, grains and green plant tissues of land origin (Castell 1942a, 1942b) it is quite apparent that seaweeds are a comparatively poor substrate for cultivating even the saccharolytic species of *Clostridium* used in these experiments. There is always the possibility, however, that special marine strains exist in the sediments of the ocean that are more adapted to growing on algae than these organisms of terrestrial origin.

As far as fish spoilage is concerned, it is the proteolytic and not the saccharolytic species that are significant, and the results indicate that these organisms cannot multiply with seaweed as a substrate.

GROWTH IN FISH

The experiments to be described are concerned only with the growth of *Clostridium* in fish under anaerobic conditions. This was accomplished by preparing the fish in vacuum packed cans or by autoclaving it in deep tubes under a soft agar gel (Spray 1936). The object has been to determine whether species of *Clostridium* will grow in fish from small inocula, under the conditions specified, without the addition of special reducing agents. The results obtained were then compared with the growth of these organisms in fish to which 0.1 per cent sodium thiogylcolate (Brewer 1940) had been added.

The experiments have been arranged in six sections: a preliminary trial using commercially canned fish and heavy inoculations from 25 cultures of *Clostridium*; inoculation of canned codfish from graded dilutions of *Cl. sporogenes*; a series of tests using graded amounts of inoculum from several species of *Clostridium* in media prepared by adding water to powdered, desiccated fish; similar tests using fish muscle from fresh and smoked fillets; the effect of adding sodium thiogylcolate to the growth of small inocula of *Clostridium* in fish; the growth of *Clostridium* in fish at low temperatures.

Except in the case of the initial tests, special significance is placed upon the number of cells in the inoculum that are necessary to initiate growth. It is a well recognized fact that many species grow under adverse conditions from a mass inoculation, whereas single cells or a relatively small number of cells find it impossible to initiate growth (Reed and Orr 1943).

Mass Inoculation of Canned Sardines and Mackerel

Commercially prepared cans of sardines and mackerel were incubated at 37°C. for one week and showed no swelling. They were then heated in boiling water, aseptically punctured, inoculated with 0.2 ml. of 48-hour broth cultures, soldered up, cooled and then left at 37°C. to incubate. As soon as a can showed

signs of swelling it was removed from the incubator and the contents examined for the presence of typical organisms.

The results (table II) would indicate that with sufficient inoculum and a partial vacuum, many common species of *Clostridium* grow in mackerel and sardines. The exceptions to this are some of the true butyric-acid forming species, which either grow slowly or not at all, as well as *Cl. sphenoides* and *Cl. difficile*.

TABLE II. Growth of various species of *Clostridium* in canned mackerel and sardines. Swelling of cans: Before 48 hr., ++++; between 48 and 72 hr., +++; between 72 hr. and 7 dy., ++; between 36 and 44 dy., +; none up to 44 dy., −.

No.	Name	Growth in canned sardines	Growth in canned mackerel
1	*Cl. novyi*	+++	++++
2	" *sporogenes*	+++	++++
3	" "	+++	++++
4	" "	++++	++++
5	" *aerofoetidum*	++++	++++
6	" *putrificum*	++++	++++
7	" *histolyticum*	++++	++++
8	" *multifermentans*	+++	++++
9	" *septicum*	++++	++++
10	" *thermosaccharolyticum*	++++	++++
11	" *tertium*	++++	+++
12	" *botulinum*—G—1	+++	+++
13	" " type A	+++	+++
14	" " " B	+++	+++
15	" " " C	+++	+++
16	" *tetani*	+	+++
17	" *welchii*	+++	++
18	" "	++	++
19	" *paraputrificum*	−	−
20	" *butyricum*	++	++
21	" "	++	+++
22	" "	++	++
23	" "	−	−
24	" *difficile*	+	−
25	" *sphenoides*	−	+
26	" *pasteurianum*	−	−

CLOSTRIDIUM SPOROGENES IN CANNED CODFISH

One-pound cans of cod were treated as described above and inoculated in triplicate from a series of dilutions made from a culture of *Cl. sporogenes*. From these same dilutions one ml. inoculations were made into Brewer's medium (1940) and a beef-liver-soy-bean-dextrose medium. They were incubated at 37°C.

At 72 hours, growth had occurred in the Brewer's medium and the liver medium in all tubes inoculated with from 1 to 10^{-9} ml. of the inoculum but

not with 10^{-10} ml. This would indicate that the inoculum had approximately a billion cells per ml.

Growth of the organisms in the canned fish appeared to progress more slowly, especially from the higher dilutions. At 72 hours only those cans inoculated with ten million or more cells showed any swelling, but after seven days with two exceptions all cans containing from 1 to 10^{-9} ml. of inoculum had become hard swells. Microscopical examination revealed typical rods, sporangia and spores in the swelled cans.

This would indicate that *Cl. sporogenes* will grow in canned codfish from relatively small inoculations, but that growth from small inocula is retarded four to five days when compared with growth from similar inocula in Brewer's medium.

Growth in Substrates Prepared from Desiccated Fish

Cod, skate, hake and pollock were carefully desiccated and powdered, both from the fillets and from the whole gutted fish. These, together with powdered lobster and fish meal, were made into culture media by suspending 5 grams in 100 ml. of a 0.1 per cent agar solution and autoclaving in deep tubes. The pH of these sterilized unbuffered fish products ranged between 5.7 and 7.0, with the majority about 6.8.

These were inoculated with serial dilutions made from the organisms listed in table II. The combined results of all these tests gave essentially the same picture but are too numerous to be recorded here. Table III gives the results of a typical set of tests.

Table III. Highest dilutions from pure cultures of *Clostridium* that produce growth in fish materials and standard anaerobic media. Tests made in triplicate; the figures given indicate at least two of the three tubes are positive. 0 means no growth in tubes inoculated with 0.1 ml. of culture or less.

Organism	Brewer's medium	Corn-liver mash	Cod	Pollock	Skate	Hake	Lobster
Cl. botulinum (A)	10^{-7}	10^{-7}	10^{-6}	10^{-7}	10^{-8}	10^{-3}	10^{-7}
" " (B)	10^{-8}	10^{-7}	10^{-8}	10^{-7}	10^{-7}	10^{-7}	10^{-8}
" " (C)	10^{-6}	10^{-5}	10^{-6}	10^{-7}	10^{-7}	10^{-3}	10^{-5}
" *sporogenes*	10^{-8}	10^{-6}	10^{-6}	10^{-6}	10^{-7}	10^{-5}	10^{-6}
" *putrificum*	10^{-8}	10^{-6}	10^{-8}	10^{-8}	10^{-8}	10^{-6}	10^{-7}
" *welchii*	10^{-7}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-5}	10^{-3}
" *butyricum*	10^{-6}	10^{-8}	10^{-1}	0	10^{-1}	0	10^{-1}
" *pasteurianum*	10^{-7}	10^{-9}	10^{-1}	10^{-1}	10^{-2}	0	10^{-2}

The proteolytic and "stinker" types of *Clostridium* grew well in the fish, fish meal and lobster media, and could initiate growth from as few cells as were required in Brewer's medium, liver and other standard anaerobic media. In almost every case, however, in the higher dilutions, growth occurred in the fish materials from several days to a week later than in Brewer's medium.

The saccharolytic types (*butyricum* and *pasteurianum*) grew poorly or not at all in the fish media. In a few instances where the inoculum contained over 10^{-6} cells, feeble growth occurred in lobster and more rarely in fish. In many tests, up to 10^{-7} cells produced no growth. With the probable exception of hake, there was very little difference in the rate of growth or the size of inoculum required, in media made from fish meal, lobster or the various fish that were used.

Growth in Fresh and Smoked Cod Fillets

Both fresh and smoked cod fillets were ground up with twice their weight of water, autoclaved in deep tubes and inoculated with dilutions made from *Cl. botulinum* (type A), *Cl. sporogenes*, *Cl. putrificum*, *Cl. tertium*, and *Cl. welchii*. Table IV shows the highest dilutions initiating growth with each of these cultures compared with growth from similar inoculations in Brewer's medium.

Table IV. Highest dilutions from pure cultures of *Clostridium* that produce growth in Brewer's medium and in fresh and smoked cod filkets. Tests made in triplicate; the figures given indicate that at least two of the three tubes were positive.

Organism	Brewer's medium	Fresh cod	Smoked cod
Cl. botulinum (A)	10^{-8}	10^{-7}	10^{-1}
" " (C)	10^{-5}	10^{-6}	10^{-4}
" *sporogenes*	10^{-9}	10^{-7}	10^{-2}
" *putrificum*	10^{-4}	10^{-7}	10^{-8}
" *tertium*	10^{-6}	10^{-7}	10^{-7}
" *welchii*	10^{-6}	10^{-6}	10^{-6}

Effect of Sodium Thioglycolate

Codfish and lobster were prepared in deep tubes with and without the addition of 0.1 per cent sodium thioglycolate and inoculated with *Cl. sporogenes*, *Cl. putrificum*, and *Cl. botulinum* (type A). The results of these tests indicated that the addition of this salt neither hastened the growth nor extended the limit to which the cultures could be diluted and still obtain growth.

Effect of Reduced Temperature

Deep tubes containing ground cod fillets and ground lobster meat were inoculated with 10 cultures of *Clostridium*, (*botulinum* types A, B and C, *sporogenes*, *putrificum*, *novyi*, *tetani*, *welchii*, *aerofoetidum* and *histolyticum*) and incubated at both 25° and 3°C. At the higher temperatures all cultures in the fish and lobster showed indications of growth at 12 days; these included gas production, characteristic odours and blackening of the tissue by the H_2S producers. After three months at 3°C. none of the cultures showed any of these characteristic changes, and microscopical examination gave no evidence of extensive proliferation of cells.

Summary and Discussion

These observations all tend to show that under anaerobic conditions, most of the proteolytic and "stinker" species of *Clostridium* grow well from small

inocula in common marine fish including cod, hake, pollock and skate. Similar results were obtained with lobster and fish meal.

Growth in fish materials was frequently delayed in starting, especially from small inocula, but ultimately it grew from inoculations as small as those producing growth in liver or Brewer's medium.

Organisms of the so-called "true" butyric acid formers, including *Cl. butyricum* and *Cl. pasteurianum*, grow very meagrely or not at all in these fish materials. This is of interest, owing to the fact that the only species that have so far been isolated from ocean sediments and from sea-water away from terrestrial contamination are saccharolytic species similar to *Cl. pasteurianum*. None of these organisms tested grew in fish held at 3°C. This is not unexpected as the species of *Clostridium* as a whole do not grow well at low temperatures; many are thermophilic and most have an optimum temperature between 30° and 40°C. However, Bergey (1939, p. 767) states that *Cl. roseum* will grow at 8°C., and Jensen (1940, p. 80) found *Cl. putrefaciens* growing in pork held at 3°C. Certainly those species growing in the mud sediments at the bottom of the ocean will grow at temperatures near freezing, but it has yet to be shown that these are of any significance in the spoilage of fish.

It would appear from this that the low temperature at which fresh fish is held is one factor which reduces the incidence of organisms in the genus.

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Effect of Salting and Smoking on Survival and Growth of *Clostridium* in Fish.

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ABSTRACT

The amount of salt necessary to inhibit the growth of *Clostridium* in fish increases with the size of inoculum. No growth occurred when 10 per cent salt was added.

Salting and smoking destroy the vegetative cells, but not the spores of *Cl. sporogenes*. The spores in the brine or on the surface of salted fish remain dormant, but viable, for long periods.

An enormous amount of work has already been done on the influence of sodium chloride on the growth and activity of microorganisms. However, very little information is available regarding the fate of non-halophilic bacteria in salted fish products. As the effect of salt on bacteria is influenced greatly by the substrate to which it is added (Tanner 1944) it seemed of particular interest to find out definitely the effect of increasing amounts of salt on the growth and survival of spore-forming anaerobes in fish products.

Next to freezing and salting, smoking is the procedure most common in the processing of many common fish. Relatively little is known of the effect of smoking on the microflora of fish. The action of smoke on *Clostridium* is of added interest in that some fish are smoked prior to being canned.

Effect of Salt Concentration on Growth of Cl. sporogenes

Hake, cod, pollock and lobster muscle were autoclaved in deep tubes in a 0.1 per cent agar solution. Sodium chloride was added to give 0, 1, 3, 5, 8 and 10 per cent concentrations. These were inoculated with dilutions of from 1 to 10^{-9} from actively growing cultures of *Cl. sporogenes*, *Cl. botulinum* (type A) and *Cl. putrificum*, and incubated at 37°C. Table I gives the highest dilutions showing growth up to 10 days' incubation.

One per cent salt had no effect, either on the rate of growth or the extent to which a culture could be diluted and still obtain growth. In some cases three per cent prevented growth in the higher dilutions and retarded it in the lower dilutions. Five per cent had a still stronger inhibiting and retarding action. At eight per cent there was no growth except where the inoculum contained millions of cells. No growth occurred in any sample containing ten per cent salt.

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TABLE I. Highest dilutions of *Cl. putrificum*, *Cl. sporogenes*, *Cl. botulinum* growing in fish and lobster containing 0, 1, 3, 5, 8 and 10 per cent sodium chloride.

Percentage sodium chloride	*Cl. putrificum*				*Cl. sporogenes*				*Cl. botulinum*			
	Hake	Lobster	Pollock	Cod	Hake	Lobster	Pollock	Cod	Hake	Lobster	Pollock	Cod
0	10^{-9}	10^{-6}	10^{-8}	10^{-7}	10^{-3}	10^{-8}	10^{-9}	10^{-6}	10^{-7}	10^{-6}	10^{-8}	10^{-6}
1	10^{-9}	10^{-5}	10^{-8}	10^{-8}	10^{-7}	10^{-8}	10^{-9}	10^{-7}	10^{-6}	10^{-5}	10^{-8}	10^{-6}
3	10^{-7}	10^{-6}	10^{-2}	10^{-4}	10^{-7}	10^{-1}	10^{-9}	10^{-6}	10^{-2}	10^{-5}	10^{-1}	10^{-3}
5	10^{-3}	10^{-6}	10^{-1}	10^{-5}	10^{-3}	10^{-2}	10^{-1}	10^{-4}	1	10^{-4}	1	1 ?
8	10^{-1}	10^{-1}	0	1 ?	1	10^{-1}	0	1 ?	0	0	0	1 ?
10	0	0	0	0 ?	0	0	0	0	0	0	0	0 ?

In the salt-free tubes the fish or lobster muscle turned a dark colour as bacterial activity progressed. Concentrations of salt above three per cent either eliminated or greatly reduced this colour change.

EFFECT OF SALTING ON SURVIVAL OF CL. SPOROGENES

Pieces of cod fillets, 50 to 60 grams, having approximately the same size and shape, were immersed for 10 seconds in a suspension of *Cl. sporogenes* made from a 72-hour culture. They were immediately salted down in a large glass container, using three parts of fish to one of salt. Periodically, one or more pieces of fish were removed from the brine and tested for the number of viable spore-forming anaerobes they harboured. This was done by thoroughly macerating the fish in a Waring blendor and making dilutions in Reed's modification of Brewer's medium (Reed and Orr 1943). After 10 days' incubation these cultures were heated to 80°C. for five minutes and then transferred to fresh thioglycolate medium. Microscopical examinations were made on the two sets of media for the presence of typical clostridial types of sporangia. Observations were also made on the type of growth and the characteristic odour produced in the medium. As no spore-forming anaerobes were found on the fish before the cultures were added, it was considered that this was sufficient evidence to indicate the presence of *Cl. sporogenes*.

The results of these tests are as follows: The fresh unsalted fish did not have any *Cl. sporogenes* or other clostridium-shaped spore-formers present. The suspension of cells used as the inoculum had approximately 10,000,000 cells per ml. The inoculated but unsalted fish had about 100,000 per g. At 24 hours after salting these 100,000 had been reduced to somewhere between 10 and 1,000 cells per g. For the next 360 days there was relatively little change. Typical anaerobic spore-formers, producing the characteristic putrid odour and cultural characteristics of *Cl. sporogenes*, were isolated from the fish after one year's submersion in concentrated brine.

By heating the fish to 75 to 80°C. for ten minutes it could be shown that the organisms which remained viable in the brine were in the spore form.

These results would indicate the following general conclusions: In the initial cell suspension in which the fish was dipped, approximately 99 per cent

of the cells were vegetative. The initial immersion in the brine destroyed all the vegetative cells, but the spores remained viable although inactive for at least one year on the fish in the concentrated brine solution.

After one year, pieces of fish were aseptically removed from the brine and allowed to dry in sterile, covered, glass containers at room temperature. After 40 days they were tested for the presence of *Cl. sporogenes* and these organisms were found present in unreduced numbers. Triplicate dilutions of the dried fish in thioglycolate medium yielded typical anaerobic spore-formers in all tubes down to the 10^{-2} dilution and in two out of three in 10^{-3} dilution.

Effect of Smoking on Survival of Cl. sporogenes

Pieces of cod fillet were contaminated with *Cl. sporogenes* in a manner similar to that described in the previous section. One-half of them were then immersed in a 20 per cent brine solution for 10 minutes. Both lots were then smoked in the Station's smoke-house for two hours. This corresponds to what the fishing trade considers to be a moderately smoked fish. The smoked fish was stored for seven days at approximately 5 to 7° and then transferred to 25°C.

During these procedures estimations of the numbers of vegetative and spore forms of *Cl. sporogenes* were made as described in the section on salting.

The results obtained were as follows: the original suspension contained approximately 1,000,000 cells per ml., and of these 10 per cent were spores. The dipped, but untreated fish had approximately 10,000 per g. One and one-half hours after smoking, the unbrined fish had only 1,000 per g. In the case of the salted fish, the brine alone reduced the count to 1,000 per g. and the subsequent smoking made no further change. These counts remained the same in both lots of fish throughout seven days in the ice box and then at room temperature until they were thoroughly spoiled.

DISCUSSION AND CONCLUSIONS

Tanner and Evans (1933) have shown that the salt tolerance of *Clostridium* varies with different strains of the same species and with the substrate to which it has been added. Their results as well as those in the literature reviewed by them suggest that the highest concentration of sodium chloride tolerated by *Cl. sporogenes*, *Cl. putrificum*, and *Cl. botulinum* is between 10 and 12 per cent.

The experiments reported in this paper indicate that strains of these three species did not grow in media prepared from cod, hake, lobster or pollock when the concentration of the salt was 10 per cent. Furthermore, when the concentration was reduced to 8 per cent growth occurred only when the inoculum contained very large numbers of viable cells. A similar observation was noted by Landerkin and Frazier (1937) in working with facultative halophiles isolated from cheese salt baths. They found that the amount of inoculum was a determining factor in the initiation of growth in media with a high salt content.

It is interesting to note that although 8 to 10 per cent salt checks the growth of all but gross contaminations of these organisms in fish, very much higher concentrations had no apparent effect on the spores. Owing to slight evaporation from the brine, the solutions were saturated with salt before many months

had passed, and at the end of a year large salt crystals were forming on the surface of the fish. Yet active growth was obtained in less than 24 hours when spores from this fish were inoculated into suitable media.

Moderate smoking, and even the light brining which frequently precedes smoking of some fish, had a similar effect in destroying all but the spores of *Cl. sporogenes*.

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The Nutritive Value of Marine Products

XVII. Value of B-Vitamins in Fish Flesh for Growth of Young Rats

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ABSTRACT

The fleshes tested as sources of the B-vitamins for the growth of young rats are listed in the order of decreasing value: pork, beef, white spring salmon, halibut, lemon sole, and lingcod. Pork flesh permitted a maximal rate of growth. The principal vitamin deficiencies found in the other fleshes are indicated in decreasing order of magnitude: beef—thiamine, riboflavin; white spring salmon—riboflavin, thiamine; halibut—riboflavin and pantothenic acid, thiamine; lemon sole and lingcod—thiamine, riboflavin, pantothenic acid.

During the last ten years there have been developed with varying degrees of success numerous chemical and microbiological assay methods for a number of the components of the B-vitamin complex. Although such advances have made the need for biological determinations less imperative, nevertheless, as Oser, Melnick and Hochberg (1945) have pointed out elsewhere, the biological method enjoys a unique advantage since it measures the available or potentially effective portion of the vitamin content of the source under examination. The chemical and microbiological methods on the other hand are designed to estimate the total amount of each vitamin present. The value of the latter data is unquestioned. It must be emphasized, however, that there exists no necessary relationship between the levels of vitamins so determined and the physiological availability of these vitamins. As has been found for some of the minerals, accompanying foods have also a marked influence on the availability of certain of the vitamins. There is for example the well known action of raw egg-white which renders biotin unavailable to the body (Sydenstricker, Singal, Briggs, DeVaugh and Isbell 1942). Certain types of live yeast when incorporated into thiamine-rich diets lower the amount of this vitamin available to the organism (Ness, Price and Parsons 1946). The role of thiaminase, present in the raw flesh of certain fish, in the destruction of thiamine and the production of a deficiency of this vitamin may also be recalled (Green, Carlson and Evans 1941). Brown, Thomas and Bina (1946) in a paper on the determination of nicotinic acid concluded that "the drastic treatment required for the liberation of nic-

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otinic acid from natural products is such as to cast doubt on the availability of large proportions of these values in human nutrition". They further believe that a comprehensive biological assay of food materials for available nicotinic acid values would be justified. The experiments herein described constitute an attempt to rate in a general way the value of different fleshes as sources of the B-vitamins for growth. Where certain of the fleshes have been found to be deficient an attempt has been made to determine wherein the deficiency lay. This work was intended to be a preliminary to more detailed study of the B-complex in fish flesh.

At the suggestion of Dr. N. M. Carter, Director of the Pacific Fisheries Experimental Station, four commercially important types of fish were chosen for this study: lingcod (*Ophiodon elongatus*), halibut (*Hippoglossus stenolepis*) lemon sole (*Parophrys vetulus*), and white spring salmon (*Oncorhynchus tshawytscha*). Other flesh preparations included for comparative purposes were those of pork and beef.

EXPERIMENTAL

The flesh samples were processed as described in a previous paper (Beveridge 1946) except that the minced flesh was dehydrated for about two hours at a temperature of not more than 40°C. and finally to a moisture content of approximately 6% at about 32°C. The total drying time varied between 8 and 10 hours. The air used in the drying operation was dried by passing it through a brine coil. The temperature of the circulating medium was −29 to −31°C. At every stage, attempts were made to shield the materials from light. These alterations in technique were made in order to minimize destruction of B-vitamins. Waisman and Elvehjem (1941) have shown that under the conditions used by them for making dried meat samples little or no destruction of vitamins took place. The method used by these workers consisted of spreading the minced meat on large pans and circulating warm air over the latter at a temperature of 35 to 50°C. The milder conditions utilized in the present study should therefore be at least as efficacious in preserving the vitamin content of the substances under investigation. The pork and beef flesh preparations were obtained by processing definite proportions of all the wholesale cuts and were thus roughly representative samples of the respective carcasses. In the two feeding trials described below fresh preparations were made for each trial. As a necessary preliminary task moisture and fat determinations were carried out on each sample. The diets were then made up on the basis of these figures to contain the same amount of moisture-free material and to be of isocaloric value. The rats were of the Wistar strain and in every case they were divided amongst the different groups according to weight, sex, and litter. They were housed in a room at a controlled temperature of 20 to 22°C. in individual cages having ¼ inch-mesh screen floors.

The basal diet was of the following composition: casein (Labco brand, fat-free, vitamin-free) 20%, sucrose 62%, beef dripping 6%, Mazola oil 4%, salts (Beveridge and Lucas 1945) 5%, agar (in first feeding trial only) 2%, celluflour (in second feeding trial only) 2%, cod liver oil (Mead's 1800 I.U. of vitamin A per g. and 175 I.U. of vitamin D per g.) 1%. Vitamin supplements

for control diet A consisted of 5% yeast and 1% Lilly's liver concentrate. These materials were incorporated in place of an equal amount of sucrose. Control diet B contained 0.3% choline chloride and 1% of a mixture of powdered sugar and crystalline vitamins made up so that each gram contained 500γ thiamine hydrochloride, 400γ riboflavin, 200γ pyridoxine hydrochloride, 1000γ calcium pantothenate, and 1000γ nicotinamide.

FEEDING TRIAL 1

The rats, males and females, on reaching 50 ± 2½ g. in weight, were placed on the basal diet for 14 days to deplete them of their stores of B-vitamins. An average weight loss of several grams resulted in most cases from this pre-

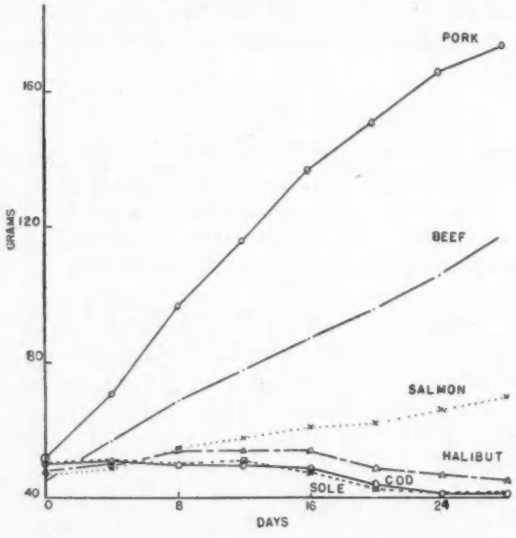

FIGURE 1. Average growth curves of males on diets in which the B-vitamins were supplied by different flesh preparations at a dietary level of 20%.

liminary treatment and where necessary some slight rearrangements were made in the various groups to make them as nearly alike as possible. Five animals were placed on each test diet and the flesh preparations were incorporated into the diets at 20 and 8% levels in place of similar amounts of sucrose. The diets, which were given *ad libitum*, were fed for 28 and 42 days respectively. Five rats were retained on the basal diet to serve as a control to demonstrate the inadequacy of the diet when no vitamins were supplied. Diet A was included to show that when B-vitamins were supplied in adequate amounts, normal growth rates resulted. Diet B was fed to show the rate of growth obtained when only the five crystalline vitamins, mentioned previously, were given.

The average growth curves for the male rats, and female rats on the 20% flesh supplements are presented in figures 1 and 2 respectively. Pertinent data are submitted in table I.

TABLE I. Gains in weight induced in young rats by diets in which the B-vitamins were supplied by 20% of different flesh preparations.

Source of B-vitamins	No. of surviving rats	Sex	Av. init. wt. (g.)	Av. final wt. (g.)	Av. gain in wt. (g.)	Av. daily gain in wt. (g.)	Av. daily food intake (g.)
Pork	1	M	51.1	173.9	122.8	4.4	10.4
	3	F	51.2	167.3	116.1	4.2	10.8
Beef	2	M	46.3	119.2	72.9	2.6	6.7
	3	F	49.4	105.7	56.3	2.0	6.5
White spring salmon	2	M	48.5	71.8	23.3	0.8	4.4
	3	F	51.3	85.4	34.1	1.2	5.1
Halibut	2	M	49.4	46.4	−3.0	−0.1	3.5
	3	F	49.8	54.5	4.7	0.2	3.6
Lemon sole	*1	M	52.5	44.2	−8.3	−0.3	2.6
	*2	F	50.3	39.8	−10.5	−0.4	2.2
Lingcod	*1	M	53.1	43.6	−9.5	−0.3	1.7
	3	F	48.4	40.6	−7.8	−0.3	2.5
(A) 5% yeast + 1% liver conc.	3	M	50.2	212.7	162.5	5.8	12.8
	2	F	43.5	136.5	93.0	3.3	9.3
(B) 1% vitamin mixture	3	M	48.3	159.0	110.7	4.0	10.9
	2	F	46.5	129.5	83.0	3.0	9.3

*One rat died.

Pork flesh proved to be by a wide margin the best source of the B-vitamins. Beef flesh proved to be a mediocre source but better than the white spring salmon preparation. Halibut, lingcod, and lemon sole were of negligible value in supplying the B-vitamins.

The rats on diets in which the B-complex was supplied by flesh at 8% levels, with the exception of those on the pork flesh ration and one individual on the beef flesh diet, all lost weight and died before the end of the experiment. The animals on the diet containing 8% pork flesh grew almost as fast as did those on the diet containing 20% beef flesh. The respective gains for the first 28 days on the test diets were for the males 2.6 and 1.8 g. per day, and for the females 1.5 and 2.0 g. per day. The animals retained on the basal diet all died within 48 hours of each other on about the eighteenth day of the test period. This result indicated that no significant amounts of the B-vitamins were contained in the basal diet. The males on the adequate control diet A gained almost 6 g. per rat per day, and the females gained between 3 and 4 g. per day. Slightly lower gains were recorded for the animals on diet B in which the only vitamins supplied were those contained in a vitamin-sugar mixture the dietary level and

composition of which has been previously mentioned. It therefore appears that under these conditions vitamins other than those supplied are required to evoke a maximal growth response.

FEEDING TRIAL 2

This experiment, which was carried out about a year after feeding trial 1, was designed first of all to see whether or not the results of the previous work

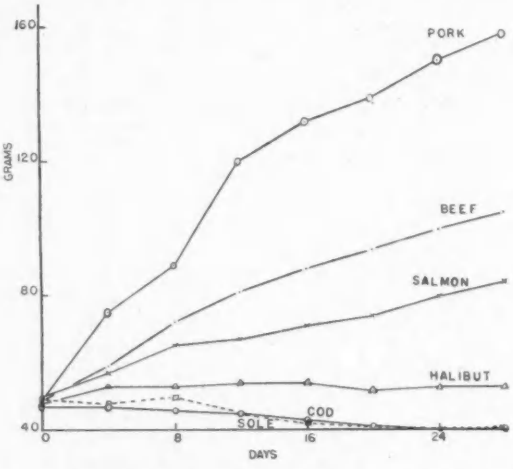

FIGURE 2. Average growth curves of females on diets in which the B-vitamins were supplied by different flesh preparations at a dietary level of 20%.

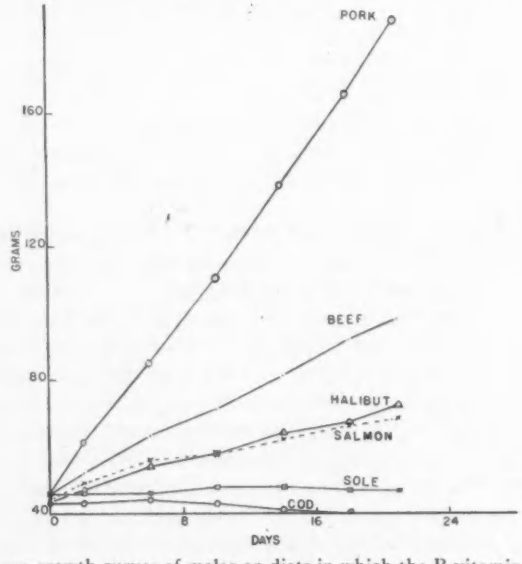

FIGURE 3. Average growth curves of males on diets in which the B-vitamins were supplied by different flesh preparations at a dietary level of 20%.

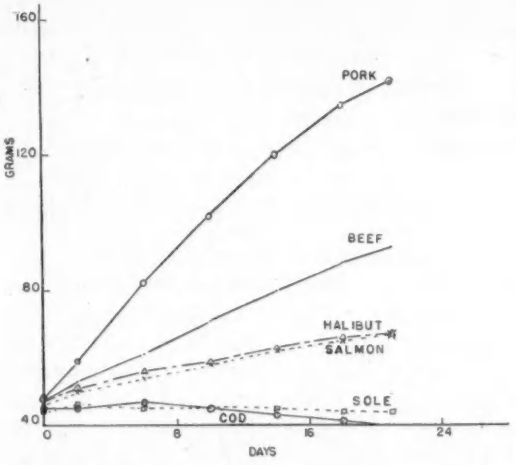

FIGURE 4. Average growth curves of females on diets in which the B-vitamins were supplied by different flesh preparations at a dietary level of 20%.

could be duplicated, and secondly to determine in which vitamins the flesh preparations were deficient. The conditions of the experiment were exactly as described for feeding trial 1 except that the test period was 21 days, five males and five females were placed on each diet, and the flesh preparations were tested only at the 20% level.

The results obtained confirmed those derived from feeding trial 1. The similarity of the growth curves is evident from a comparison of figures 1 to 4

TABLE II. Gains in weight induced in young rats by diets in which the B-vitamins were supplied by 20% different flesh preparations.

Source of B-vitamins	No. of rats	Sex	Av. init. wt. (g.)	Av. final wt. (g.)	Av. gain in wt. (g.)	Av. daily gain in wt. (g.)	Av. daily food intake (g.)
Pork	5	M	47.6	185.1	137.5	6.5	13.0
	5	F	49.8	142.3	92.5	4.4	11.5
Beef	5	M	45.8	99.6	53.8	2.6	6.6
	5	F	46.9	93.7	46.8	2.2	7.0
Halibut	5	M	44.4	73.2	28.8	1.4	4.8
	5	F	46.4	68.4	22.0	1.0	4.8
White spring salmon	5	M	46.6	69.6	23.3	1.1	4.7
	5	F	48.1	69.5	21.4	1.0	4.9
Lemon sole	5	M	47.9	47.5	−0.4	0.0	3.4
	5	F	45.5	44.8	−0.7	0.0	3.7
Lingcod	5	M	44.7	37.6	−7.1	−0.3	2.8
	*4	F	47.4	39.0	−8.4	−0.4	3.1

*One rat died.

and demonstrates that the results obtained are reproducible and therefore valid in a relative sense at least. The growth induced by the halibut diet is definitely greater than that observed in the first experiment. The relevant data of the feeding experiment are given in table II.

It is interesting to note that the diet containing pork flesh induced a maximal growth rate.

In order to determine approximately wherein the different flesh preparations were deficient, different combinations of the five crystalline vitamins incorporated in control diet B were injected daily for 10 days into the rats at the completion of the regular test period of 3 weeks. The same vitamin injections were given to similarly numbered males and females of each group. The series, referred to as 1—5, was comprised of five pairs, one male and one female in each pair. All rats received 50 γ thiamine hydrochloride. In addition, number one rat of each group was given 40 γ riboflavin, number two rat 25 γ pyridoxine hydrochloride, number three rat 100 γ calcium pantothenate, number four rat 100 γ nicotinic acid, and number five rat 40 γ riboflavin and 25 γ pyridoxine hydrochloride. The vitamins were dissolved or suspended in 0.9% saline so that 0.1 ml. contained the amount of each vitamin injected daily.

In addition to the animals already described being given the test diets, two male rats, termed X and Y, were placed on each diet at the end of the preliminary 14-day depletion period and given daily injections of different vitamins. The appellations given to these two series are X_1 and Y_1 for the first 21-day test period, and X_2 and Y_2 for the last 10-day test period. The X_1 rats received 50 γ thiamine hydrochloride daily, and the Y_1 rats received 40 γ riboflavin, 25 γ pyridoxine hydrochloride, 100 γ calcium pantothenate and 100 γ nicotinamide. In the 10 days following the regular test period rats termed X were given, in addition to the B_1, an additional supplement consisting of B_2, pyridoxine hydrochloride and calcium pantothenate; the rats termed Y were given, in addition to the vitamins previously given, thiamine hydrochloride. The data concerning weight gains and food consumption of the rats during the period in which the vitamin supplements were administered are shown in tables III—VIII.

DISCUSSION

An examination of the daily gains in weight (see tables III—VIII) brought about in the rats by different combinations of injections gives some idea wherein the worst vitamin deficiencies lie. The fleshes will be considered in the order of decreasing value as a source of the B-complex for growth.

Pork Flesh

In no case did the addition of any combination of vitamins bring about an increased rate of growth (table III) and since the unsupplemented flesh induced growth comparable to that observed in the animals on the positive control diet (A) (see tables I and II), it may be assumed that a diet containing 20% pork flesh supplies adequate amounts of B-vitamins to promote a maximal rate of growth in rats in the period under examination.

Beef Flesh

When thiamine was added to rat X_1 (table IV) the rate of growth was about the maximum. On the other hand the addition of all the other vitamins to rat Y_1 brought about no increase in growth over that recorded for the animals on the unsupplemented diet (table I, II). This result indicates that thiamine is the chief vitamin limiting growth in the diet containing beef flesh. In the series 1—5 (table IV) in which thiamine was given along with different com-

Table III. Effect of various vitamin supplements on the ability of a diet containing 20% pork flesh as a source of B-vitamins to promote growth in young rats.

Rat no.	Vitamin supplement	Sex	Init. wt. (g.)	Final wt. (g.)	Gain in wt. (g.)	Daily gain in wt. (g.)	Daily food intake (g.)
1	B_1+B_2	M	180.2	223.9	43.7	4.4	15.9
		F	144.5	161.1	16.6	1.7	13.0
2	B_1+pyridoxine HCl	M	187.7	244.2	56.5	5.7	18.8
		F	140.6	156.6	16.0	1.6	13.1
3	B_1+Ca pantothenate	M	179.0	226.0	47.0	4.7	18.6
		F	143.9	163.0	19.1	1.9	12.3
4	B_1+nicotinamide	M	182.5	231.2	48.7	4.9	14.9
		F	124.9	146.5	21.6	2.2	11.5
5	B_1+B_2+pyridoxine HCl	M	196.0	237.2	41.2	4.1	16.9
		F	157.5	172.2	14.7	1.5	12.2
X_2	As above + Ca pantothenate	M	174.3	225.1	50.8	5.1	17.3
Y_2	As above + nicotinamide	M	181.7	219.4	37.7	3.8	16.2
X_1	B_1	M	51.3	174.3	123.0	5.9	11.7
Y_1	B_2+pyridoxine HCl + Ca pantothenate + nicotinamide	M	47.6	181.7	134.1	6.4	14.3

binations of the other vitamins, essentially the same gain was recorded for all with the exception perhaps of those rats to which riboflavin was also given. It therefore appears that the beef flesh preparation used was definitely deficient in thiamine and perhaps slightly deficient in riboflavin.

Salmon Flesh

An examination of table V reveals that neither the B_1 given to rat X_1 nor the other four crystalline vitamins given to rat Y_1 evoked a maximal growth response, although in the latter case the growth rate approached normal. This indicated that the salmon flesh diet constituted a fair source of B_1 and that

some one or all of the other vitamins were slightly deficient. However, the results of the series, 1—5, X_2, Y_2, indicated that only when B_1 and B_2 were given together was a maximal rate of growth obtained and the rate of growth was as great when only B_1 and B_2 were given as when all the vitamins were administered. These data reveal that the principal deficiency in salmon flesh is riboflavin and that, although it is a relatively good source of thiamine, the amount in a diet containing 20% salmon flesh is not quite sufficient to promote a maximal rate of growth.

TABLE IV. Effect of various vitamin supplements on the ability of a diet containing 20% beef flesh as a source of B-vitamins to promote growth in young rats.

Rat no.	Vitamin supplement	Sex	Init.wt. (g.)	Final wt. (g.)	Gain in wt. (g.)	Daily gain in wt. (g.)	Daily food intake (g.)
1	$B_1 + B_2$	M	73.9	140.8	66.9	6.7	11.2
		F	104.6	143.5	38.9	3.9	12.0
2	B_1 + pyridoxine HCl	M	110.0	166.3	56.3	5.6	12.9
		F	129.4	158.5	29.1	2.9	12.6
3	B_1 + Ca pantothenate	M	86.4	138.9	52.5	5.3	10.6
		F	110.5	154.7	44.2	4.4	13.7
4	B_1 + nicotinamide	M	123.0	181.0	58.0	5.8	13.4
		F	48.2	85.3	37.1	3.7	8.3
5	$B_1 + B_2$ + pyridoxine HCl	M	104.9	174.3	69.4	6.9	14.1
		F	75.6	88.4	12.8	1.3	11.0
X_2	As above + Ca pantothenate	M	151.2	206.8	55.6	5.6	12.3
Y_2	As above + nicotinamide	M	92.8	148.2	55.4	5.5	13.0
X_1	B_1	M	41.1	151.2	110.1	5.2	10.0
Y_1	B_2 + pyridoxine HCl + Ca pantothenate + nicotinamide	M	53.7	92.8	39.1	1.9	6.5

HALIBUT

The addition of B_1 alone (X_1, table VI) brought about only a slight increase in the rate of growth over that observed on the animals given the unsupplemented flesh diet. The injection of the four other vitamins (Y_1) elicited weight gains which were just slightly subnormal, thus indicating that the halibut preparation was a fair source of vitamin B_1. In the series 1—5 injections of B_1 plus any one of the other vitamins brought about increased gains in weight which were definitely subnormal compared to the maximal growth rate. When B_1 plus B_2 were given, the gains were greater than was obtained with any other combination of two vitamins. Since the addition of pyridoxine to a combination of B_1 plus

B_2 brought about no increase in weight gains, it may be assumed that halibut flesh is an adequate source of this vitamin.

When calcium pantothenate was added to this combination a definite increase in the rate of growth was observed, indicating that this vitamin had been a limiting factor in growth (see rat X_2). When nicotinamide was added to the combination (see rat Y_2) a further slight increase of doubtful

TABLE V. Effect of various vitamin supplements on the ability of a diet containing 20% salmon flesh as a source of B-vitamins to promote growth in young rats.

Rat no.	Vitamin supplement	Sex	Init. wt. (g.)	Final wt. (g.)	Gain in wt. (g.)	Daily gain in wt. (g.)	Daily food intake (g.)
1	$B_1 + B_2$	M	79.7	146.4	66.7	6.7	11.4
		F	74.9	134.7	59.8	6.0	12.1
2	B_1 + pyridoxine HCl	M	80.6	122.7	42.1	4.2	8.7
		F	72.2	102.1	29.9	3.0	8.0
3	B_1 + Ca pantothenate	M	73.1	109.5	36.4	3.6	8.0
		F	75.4	109.3	33.9	3.4	8.9
4	B_1 + nicotinamide	M	62.4	97.4	35.0	3.5	7.5
		F	62.3	84.1	21.8	2.2	6.4
5	$B_1 + B_2$ + pyridoxine HCl	M	53.8	104.1	50.3	5.0	8.2
		F	62.5	97.6	35.1	3.5	9.1
X_2	As above + Ca pantothenate	M	92.3	153.6	61.3	6.1	12.3
Y_2	As above + nicotinamide	M	128.3	182.5	54.2	5.4	15.4
X_1	B_1	M	44.5	92.3	47.8	2.3	6.2
Y_1	B_2 + pyridoxine HCl + Ca pantothenate + nicotinamide	M	37.1	128.3	91.2	4.3	8.7

significance was observed. One may therefore conclude that the halibut flesh seemed to be adequate in pyridoxine and perhaps in nicotinamide, whereas thiamine, riboflavin, and pantothenic acid were present in sub-optimal amounts for maximal growth.

LEMON SOLE

When only thiamine was added the rate of growth was extremely slow (rat X_1, table VII) but without it the rats actually lost weight (table II). The addition of all the other vitamins without thiamine resulted in a loss of weight, thus indicating the low level of the latter vitamin in this preparation. The addition of B_1 and B_2 evoked a rate of growth which was only slightly subnormal.

The two chief limiting factors in growth therefore appear to be the deficiency of these two vitamins in the lemon sole flesh. The injection of pyridoxine along with these two vitamins caused no apparent difference in growth rate and indicates that pyridoxine is probably present in adequate amounts. When calcium pantothenate was added to the latter combination of vitamins maximal growth ensued. It appears therefore that lemon sole flesh is grossly deficient in B_1, and deficient in riboflavin and pantothenic acid. The evidence indicated that pyridoxine and nicotinamide are present in sufficient quantity.

TABLE VI. Effect of various vitamin supplements on the ability of a diet containing 20% halibut flesh as a source of B-vitamins to promote growth in young rats.

Rat no.	Vitamin supplement	Sex	Init. wt. (g.)	Final wt. (g.)	Gain in wt. (g.)	Daily gain in wt. (g.)	Daily food intake (g.)
1	$B_1 + B_2$	M	81.1	122.0	40.9	4.1	9.2
		F	59.1	96.3	37.2	3.7	8.2
2	B_1 + pyridoxine HCl	M	75.2	108.0	32.8	3.3	7.6
		F	86.2	109.0	22.8	2.3	8.4
3	B_1 + Ca pantothenate	M	71.8	104.5	32.7	3.3	7.4
		F	66.7	101.5	34.8	3.5	9.6
4	B_1 + nicotinamide	M	74.0	102.3	28.3	2.8	6.6
		F	67.2	94.3	27.1	2.7	9.4
5	$B_1 + B_2$ + pyridoxine HCl	M	64.1	105.0	40.9	4.1	8.0
		F	62.8	101.3	38.5	3.9	8.9
X_2	As above + Ca pantothenate	M	97.9	154.7	56.8	5.7	12.9
Y_2	As above + nicotinamide	M	127.4	192.6	65.2	6.5	15.9
X_1	B_1	M	51.4	97.9	46.5	2.2	7.3
Y_1	B_2 + pyridoxine HCl + Ca pantothenate + nicotinamide	M	45.2	127.4	82.2	3.9	9.1

LINGCOD

A study of tables VII and VIII shows the existence of a marked similarity between the data obtained for lingcod and lemon sole, and the conclusions reached for the latter may be applied also to the former.

The question of the effect of differences in the amino acid composition of the proteins of the fish, beef and pork preparations on the requirement of B-vitamins has been raised. Although it is true that the amino acid composition of these substances differs slightly, nevertheless, the possibility is extremely faint that these slight differences, superimposed on a diet already adequate in

protein, would exert any demonstrable effect on the requirement of the B-vitamins.

In every case when the five crystalline vitamins used in this study were given as a supplement to flesh diets maximal growth was obtained. This result indicates that the flesh preparations supplied adequate amounts of the other factors required to promote a maximal growth rate. That such metabolites are required may be revealed by comparing the respective gains induced by control diets A and B (cf. table I).

TABLE VII. Effect of various vitamin supplements on the ability of a diet containing 20% lemon sole flesh as a source of B-vitamins to promote growth in young rats.

Rat no.	Vitamin supplement	Sex	Init. wt. (g.)	Final wt. (g.)	Gain in wt. (g.)	Daily gain in wt. (g.)	Daily food intake (g.)
1	$B_1 + B_2$	M	48.9	88.7	39.8	4.0	7.2
		F	45.9	77.0	31.1	3.1	6.6
2	B_1 + pyridoxine HCl	M	43.7	65.1	21.4	2.1	5.8
		F	44.1	61.7	17.6	1.8	5.4
3	B_1 + Ca pantothenate	M	44.1	61.7	17.6	1.8	4.4
		F	47.7	64.4	16.7	1.7	4.9
4	B_1 + nicotinamide	M	56.4	74.6	18.2	1.8	5.0
		F	39.1	60.6	21.5	2.2	5.7
5	$B_1 + B_2$ + pyridoxine HCl	M	44.3	86.0	41.7	4.2	6.9
		F	47.2	85.6	38.4	3.8	7.7
X_2	As above + Ca pantothenate	M	81.9	140.8	58.9	5.9	11.9
Y_2	As above + nicotinamide	M	50.2	102.4	52.2	5.2	9.1
X_1	B_1	M	47.4	81.9	34.5	1.6	5.5
Y_1	B_2 + pyridoxine HCl + Ca pantothenate + nicotinamide	M	45.9	50.2	4.3	0.2	4.4

SUMMARY

The relative values of the fleshes of lingcod, lemon sole, halibut, white spring salmon, beef, and pork at the 20% level as sources of the B-vitamins have been determined twice using the growth rate of young rats as a criterion.

Pork flesh permitted a maximal rate of growth. The other fleshes evoked subnormal growth responses and are placed in decreasing order of adequacy for growth with respect to the B-vitamins: beef, white spring salmon and halibut. The feeding of lemon sole and lingcod brought about weight losses.

The principal deficiencies in the different fleshes as determined by the effects of the injection of various combinations of vitamins are indicated in

decreasing order of magnitude: beef—thiamine, riboflavin; salmon—riboflavin, thiamine; halibut—riboflavin and pantothenic acid, thiamine; lemon sole and lingcod—thiamine, riboflavin and pantothenic acid.

The group of fish flesh tested compared most unfavourably with pork and beef flesh as sources of the B-vitamins for the growth of young rats.

TABLE VIII. Effect of various vitamin supplements on the ability of a diet containing 20% lingcod flesh as a source of the B-vitamins to promote growth in young rats.

Rat no.	Vitamin supplement	Sex	Init. wt. (g.)	Final wt. (g.)	Gain in wt. (g.)	Daily gain in wt. (g.)	Daily food intake (g.)
1	$B_1 + B_2$	M	37.3	76.5	39.2	3.9	6.9
		F	42.7	71.3	28.6	2.9	6.5
2	B_1 + pyridoxine HCl	M	35.6	57.0	21.4	2.1	4.5
		F	39.5	52.3	12.8	1.3	4.3
3	B_1 + Ca pantothenate	M	39.7	57.3	17.6	1.8	5.1
		*F					
4	B_1 + nicotinamide	M	40.8	58.9	18.1	1.8	4.2
		F	38.3	51.5	13.2	1.3	4.3
5	$B_1 + B_2$ + pyridoxine HCl	M	34.4	63.3	28.9	2.9	4.9
		F	35.3	57.0	21.7	2.2	4.6
X_2	As above + Ca pantothenate	M	76.2	140.4	64.2	6.4	11.0
Y_2	As above + nicotinamide	**M					
X_1	B_1	M	39.3	76.2	36.9	1.8	4.8
Y_1	B_2 + pyridoxine HCl + Ca pantothenate + nicotinamide	M	45.5	34.9	−10.6	−0.5	3.4

*Died during the preliminary 21-day test period.
**Died during the 10-day test period.

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Experiment to Develop sea-run from land-locked Sockeye Salmon (*Oncorhynchus nerka kennerlyi*)

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ABSTRACT

Kokanee eggs from the Kootenay area were transferred to Cultus lake, hatched, reared to yearling size, marked by removal of both pelvic fins and released to proceed seaward along with the normal sockeye migration from Cultus. No returns of four-year-old adults, the most common age group for the Fraser, were obtained, but the following year 25 five-year-olds were recovered from 34% of the commercial fishery sampled and 17 adults bearing the correct mark were taken at Cultus. Ages could not be determined, therefore some or all of these may have been mismarked four-year-olds. The results indicate that sea-run sockeye can develop from kokanee populations where migration to sea is possible. It is suggested that the size difference between sockeye and kokanee is more likely due to environment than to heredity. Evidence for habit is less clear.

Two types of *Oncorhynchus nerka* (Walbaum) are commonly found in British Columbia, one the normal, anadromous form commonly called sockeye salmon (*O. nerka*) and the other a diminutive, lake-dwelling form, popularly called the little red fish or kokanee, *O. nerka kennerlyi*. The only recognizable differences are in habit and size, the kokanee tending to reside permanently in the lakes and exhibiting a decidedly slower rate of growth. Jordan and Evermann (1896) remark "We are not able to discover any structural differences between the two. We have found them breeding at the same time and in the same stream."

Ricker (1938) discovered a third type in Cultus lake to which he gave the name "residual." These are non-anadromous individuals, presumably largely if not wholly the progeny of anadromous parents and differ from the kokanee in breeding colour, time of spawning, size at maturity and sex ratio. Ricker inclines to the belief that while residuals are direct descendants of anadromous individuals, which have, for reasons not clearly defined, taken up permanent lake residence, the kokanee while perhaps originating at some time from the residuals, "now appear to be a population distinct from the latter and from the anadromous fish" (Ricker 1938, p. 217). See also Ricker (1940).

Prior to Ricker's introduction of a third and perhaps genetically intermediate type into the series, consideration had been given to the possibility that certain proportions of the progeny of kokanee spawnings in certain areas

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or in certain seasons when conditions were favourable might respond, along with normal sockeye smolts, to whatever stimuli bring about seaward migration, and become sea-run individuals again. It was thought that perhaps there were occasions when some young kokanee would revert to sea-run sockeye, even as some young sockeye presumably become lake-dwelling kokanee. If, as Ward (1932) maintains, the factor responsible for production of lake-dwelling forms or kokanee is the development of a vernal temperature "blanket" which seals, as it were, the surface strata of the lake to sockeye smolts seeking to proceed seaward by way of the lake outlet, particularly, according to Foerster (1937), those relatively smaller yearlings which tend to migrate toward the end of the season, it seemed quite possible that in certain seasons the temperature conditions might be the reverse and lead to the migration of kokanee yearlings as well.

If this situation could prevail it seemed further possible that transfer of kokanee eggs or fry from native areas to those where conditions would bring about seaward migration might result in the restoration of depleted sockeye areas and make available the immense stocks of kokanee eggs collectible in many interior lakes and not otherwise utilized.

EXPERIMENTAL PROCEDURE

To test the possibility of such practice, an experiment was commenced at Cultus lake in the spring of 1933 when a shipment of 200,000 kokanee eggs collected in the Kootenay lake area was obtained from the Nelson hatchery and placed in the Cultus lake hatchery for hatching, the fry being subsequently transferred to retaining ponds for rearing to the yearling stage.

Severe loss attended the hatching of the eggs and some mortality occurred during pond retention but in the spring of 1934 a total of 63,874 yearling kokanee was available for liberation. In order to identify the fish when taken in the commercial fishery or on their return to Cultus lake all individuals were marked by removal of the two pelvic fins. The fish were all of good size—3 to 4 inches (7.5 to 10 cm.) in length—and in order to assure that there would be no possibility of their maintaining their lake-dwelling tendencies in Cultus lake they were liberated in Sweltzer creek, the outlet stream of Cultus lake, below the migrant counting fence which barred their possible up-stream ascent to the lake.

RESULTS

The return of adults was expected in 1936, assuming that the liberated yearlings would drop down to the sea as the normal sockeye smolts from Cultus lake were doing, and remain there until their fourth year (from time of spawning, 1932). At that time notices were displayed throughout the fishery and as much publicity as possible given to the probable presence of marked sockeye among the commercial catch. Fishermen and cannery workers were urged to watch for these marked individuals and to send in the scars of the missing fins, the pertinent data and scale samples.

Only one operator, Mr. R. Nesbitt of the Glenrose Cannery, recovered what appeared to be marked specimens and submitted them for examination. However, of the four scars sent in, three were without question non-authentic

marks. In one case non-development of the pelvic bones was the cause of the apparent mark and in the other two it was apparent that the fins had suffered mutilation. Stubs of fins were present and the scars were not typical of pelvic markings. The fourth fish, a small male having the adipose lacking as well as the two pelvics, was a three-year-old individual, obviously belonging to the seaward migration from Cultus lake in 1935 of which all the migrants were marked by the removal of adipose and both pelvic fins.

Recovery of marked adults at Cultus lake was no more successful. All of the 8,359 adults reaching the counting fence below the lake (Foerster 1936a, figs. 2 and 3) were closely examined and only three were found with the kokanee mark. Of these, two were small males, presumably mismarked three-year-olds of a Cultus lake marking in 1935 while the third, a large male, bore a scar which was questionable, possibly the result of accidental mutilation.

It was concluded, therefore, that the transfer of kokanee stocks to Cultus lake and release there to form an anadromous run had proven a failure, unless the fish tended to remain in the ocean an extra year and to return in 1937 as five-year-olds. This latter conjecture proved to be the case, *in some degree*, for in 1937, sockeye with both pelvic fins only off and in their fifth year were retaken in the commercial fishery. That these fish did not experience any slower growth rate in the ocean than normal sockeye was indicated by mean measurements which showed for five-year-old males (12 individuals) a mean length of 67.8 cm. and for females (10 individuals) 64.5 cm. For four-year fish the mean lengths obtained were 59.2 cm. for males (147 individuals) and 56.6 cm. for females (203 individuals). Clemens (1938) records average lengths of 25.5 inches (64.8 cm.) and 24.4 inches (62 cm.) for Fraser river five-year males and females respectively. Similarly marked individuals were recovered at Cultus lake but the ages could not be determined.

In 1937 there were expected back, in addition to any five-year-old kokanee (two pelvic fins removed), the returns from normal sockeye marking experiments at Cultus lake in 1935 (two pelvic and the adipose fins off) and 1936 (two pelvic fins only)—in which years all migrants were marked—and observers had been stationed at certain strategic canneries in each of the fishing areas to inspect samples of the catches and to note the numbers of marked individuals occurring.

The complete records for the season's operations were:

Total sockeye reported from the commercial fishery	2,225,000
Total sockeye observed for marks	756,000
Number of marked three-year-olds observed, from marking of 1936 (two pelvics only)	311
Number of marked four-year-olds observed, from marking of 1935 (adipose and two pelvics)	379
Number of marked five-year-olds observed, from kokanee marking of 1934 (two pelvics only)	25

From approximately 34 per cent of the commercially-caught sockeye 25 marked five-year fish, presumably originating from the kokanee transfer to Cultus lake, were obtained. The best estimate of the total marked five-year-olds in the 1937 commercial fishery would be approximately 74, with fiducial limits, on a 0.95 confidence coefficient basis (Ricker 1937), ranging from 48 to 108.

At Cultus lake, there were taken in 1937 the following:

	Males	Females
Small sockeye, presumably three-year-olds, with two pelvics only off	1,583	104
Small sockeye, with one pelvic fin only or no fins removed (either poorly marked three-year-olds or strays)	138	9
Large sockeye with adipose and two pelvic fins missing (presumably four-year-olds)	401	626
Large sockeye with two pelvics only off	9	8
Large sockeye with one pelvic fin only or no fins missing (either poorly marked kokanee or normal five-year-olds from the unmarked 1934 seaward migration)	103	80

Only 17 individuals carrying the mark given to kokanee were recovered at the Cultus counting weir. These were all large adults, definitely either four- or five-year fish but since their scales were too seriously absorbed around the margins to make an accurate determination of age it was impossible to ascertain whether they were authentic five-year-old kokanees or merely mismarked four-year-old sockeye belonging to the Cultus marking of 1935, a few of which were encountered in the recoveries from the commercial fishery.

DISCUSSION AND CONCLUSIONS

From the transfer of kokanee eggs from the interior Kootenay lake area to Cultus lake and the subsequent marking and liberation of 63,874 yearlings there were recovered: (a) no four-year-old adults in the fishery in 1936; (b) no four-year-old adults returning to Cultus lake; (c) 74± five-year-old adults from the commercial fishery of 1937; (d) 17 large adults returning to Cultus lake, some or all of which might be authentic returns.

The experiment was not therefore wholly devoid of positive results. Some sea-running sockeye were produced but the number captured in the fishing areas, 0.12 per cent of yearlings liberated, was decidedly lower than for normal sockeye smolts, as determined in 1932, 1.9 per cent, and in 1933, 2.6 per cent (Foerster 1936b). The fact that the adults returned as five-year-olds rather than as normal four-year sockeye introduces a complication but this may have been an unusual phenomenon not necessarily applying rigidly to all kokanee that might take up anadromous habits.

Subsequent to the completion of this experiment Chapman (1941) has raised the question as to the possibility of kokanee populations, in lakes which have been rendered inaccessible to sockeye by construction of high dams, continuing to maintain the annual runs of sockeye to the rivers on which these dams are situated. Similar conditions arising in streams with impassable falls have frequently aroused conjecture as to the origin of the adult sockeye present.

The findings of the present Cultus lake experiment suggest that kokanee may adopt the anadromous habits of the sockeye once they have entered upon seaward migration as yearlings. To what extent, however, under normal

conditions the factors inhibiting seaward migration, either physical (Ward 1932; Foerster 1937) or genetic (Ricker 1938, 1940), happen to be inoperative upon the whole or part of the yearling kokanee of lakes containing kokanee populations is not known. It still remains conceivable that an interchange between the two populations may occur and this circumstance may explain the surprisingly sudden development of sockeye runs to certain rivers and the converse gradual diminution of runs in existing sockeye areas where lake conditions have changed.

If a possible interchange between sockeye and kokanee populations can take place through a "residual" stage, as Ricker (1938) indicates, or more directly, it would seem that in those lake areas where both populations occur the circumstances or factors which stimulate seaward migration of some of the sockeye and inhibit escape of others which later become residuals or kokanee must depend largely on conditions existing in the lakes each year and therefore may vary appreciably in governing the proportion of available potential migrants that actually take part in the seaward migration.

The return from the ocean of the pond-reared kokanee, after liberation in a location where they were not subject to the conditions of a lake environment, raises the question as to whether the differences between sockeye and kokanee—principally size and habit—are hereditary or environmental. The present results suggest that size cannot be hereditary since the released kokanee were indistinguishable from sockeye when returning as adults. It is further established by the frequently observed fact that sockeye when reared to adult size wholly in fresh water attain a conspicuously smaller length and weight. Environment therefore must be a strongly-influencing factor.

As far as habit is concerned the experiment has indicated that, when liberated in a stream below a lake and barred from ascending into the lake, some of the kokanee proceeded to sea and returned. What would have happened if the kokanee had been released into Cultus lake as fry, fingerlings or as yearlings is not known. What would have occurred if there had been no obstructions to the ascent of the liberated kokanee into the lake is not known. Certainly none were ever observed in the outlet stream subsequent to the planting as might have been expected if they had sought to return immediately to the lake.

SUMMARY

An experiment was undertaken at Cultus lake, British Columbia, to ascertain whether the progeny of lake-dwelling kokanee salmon, *O. nerka kennerlyi*, could be used to augment existing stocks of anadromous sockeye, *O. nerka*. Kokanee eggs were transferred to Cultus lake for hatching, rearing and the liberation of the yearlings under conditions which would presumably result in their migrating to sea along with normal sockeye seaward migrants. A liberation of 63,874 yearlings was made, the fish being marked by removal of both pelvic fins prior to release.

No marked adults were obtained in the cycle fourth year, 1936, either in the commercial fishery or at Cultus lake. In 1937, however, 25 individuals with both pelvic fins lacking were taken in that portion (34%) of the commercial

fishery sampled, all individuals being found to be in their fifth year. Length measurements showed them to be larger than normal four-year Cultus sockeye and well within the range for five-year-olds. Seventeen similarly marked individuals were retaken at Cultus lake but it cannot be definitely asserted that all or any of these were the return from the kokanee liberation, due to the possibility of confusion with other markings at Cultus lake in 1935 and the inability to determine the ages of the adult fish.

The results suggest that of the two distinctive differences between sockeye and kokanee—size and habit—the former cannot be hereditary and probably is environmental. For habit, the difference is less clear but environment would seem to be a strongly-influencing factor.

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Thiaminase in Aquatic Animals of Nova Scotia

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ABSTRACT

The muscle and viscera of a number of fresh water and marine animals have been tested for thiaminase activity. Residual thiamine was determined by chemical and yeast methods. The enzyme was found in a greater proportion of fresh water species and invertebrates.

In recent years an acute dietary disease known as Chastek paralysis has been found to cause serious losses on certain fox farms throughout the United States. The syndrome appears clinically as anorexia, hyperesthesia, ataxia, and spastic paralysis followed by death within 72 hours after the onset of the neurological condition (György 1942). The disease was found only on farms where fresh or frozen fish constituted 10 per cent or more of the diet and it could be experimentally reproduced in foxes fed on uncooked carp (*Cyprinus carpio*). Since either feeding of large amounts of thiamine or cooking the fish prevented the disease, Green *et al.* (1941, 1942) believed that some heat-labile substance in fresh and frozen fish effected the destruction of the thiamine therein.

Later studies (Spitzer *et al.* 1941, Wooley 1941) served to indicate the *in vitro* thiamine destroying property of carp tissues, while Sealock *et al.* (1943) showed that this so-called "fish principle" was most probably of the nature of an enzyme. More convincing evidence for this view was advanced by Krampitz and Wooley (1944) based on their observation of the liberation in a reaction mixture containing carp viscera extract and thiamine, of 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine. These substances were believed to be formed from the thiamine molecule following the enzymatic addition of water to the vitamin. Finally, Sealock and Goodland (1944) were able to employ certain competitive inhibitors in such a way that the analytical treatment of Lineweaver and Burk (1934) could be applied to follow the mechanism of thiamine destruction.

Deutsch and Hasler (1943) found the anti-thiamine factor in 15 out of 31 freshwater fish, but failed to detect the enzyme in any of 9 sea species examined. However, the factor has been stated to be present in "Atlantic herring and whiting and in Pacific mackerel" and in various varieties of clams of the *Mya arenaria* group (Melnick *et al.* 1945). Recently Leick and Ågren (1945) have discovered the principle in a number of fresh water fish of Sweden.

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Since the distribution of the thiamine-destroying enzyme is of both theoretical and practical significance, the analysis of the tissues of some aquatic animals of Nova Scotia has been undertaken to determine its presence or absence.

EXPERIMENTAL

The fish were taken from the water, frozen before the muscles developed rigor, and held in a cold room at −30°C. until analysed. They were allowed to thaw at room temperature, after which the visceral organs (minus stomach contents) and a standard portion of muscle including the skin were disintegrated separately in a Waring blendor with an equivalent weight of 2% aqueous sodium chloride. When only small amounts of tissue were available, the grinding was accomplished through the aid of a mortar, pestle and clean sand. The mixtures were divided into two portions, one being held in water at 100°C. for 30 minutes. The optimum conditions for the determination of thiaminase activity having been previously worked out by Sealock *et al.* (1943), this procedure has been followed very closely. One ml. amounts of the fish brei were transferred to a 50 ml. beaker containing 3 ml. phosphate buffer of pH 7.4 and one ml. of a solution of crystalline thiamine hydrochloride containing 100 micrograms of the vitamin per ml. Incubation was carried out in a water bath at 50°± 0.1°C. for one hour. Analysis for residual thiamine was determined in the samples of species from fresh water by the diazonium method of Melnick and Field (1939), using the technique of Sealock *et al.* (1943). Samples taken from sea fish were analysed both by the above chemical method and by the yeast fermentation assay method of Schultz, Atkin and Frey (1942), as outlined by Tauber (1943). A local strain of bakers' yeast manufactured by the Best Yeast Company, Liverpool, N.S. and specified by them to be a special strain of *Saccharomyces cerevisiae*, was used.

TABLE I. Thiaminase activity of tissues of various aquatic animals of Nova Scotia, as shown by per cent of thiamine destroyed in unheated samples by equivalent amounts of tissue.

(a) Forms from fresh water

Species tested		Chemical method						
		Muscle			Viscera			
		I	II	III	I	II	III	±
Elliptio complanatus	Mussel	16	18	31	−	−	−	+
Pomolobus pseudoharengus	Alewife	13	24	11	8	10	20	+
Salmo salar	Salmon	5	6	3	1	0	3	−
Osmerus mordax	Smelt	4	9	17	11	21	19	+
Fundulus heteroclitus	Minnow	35	54	64	47	53	62	+
Fundulus diaphanus	Minnow	61	39	57	72	74	63	+
Anguilla rostrata	Eel	0	2	0	0	3	2	−
Catostomus commersonii	Sucker	22	7	10	36	12	11	+
Ameiurus nebulosus	Catfish	19	15	31	14	23	46	+
Morone americana	White perch	0	0	0	0	5	0	−
Perca flavescens	Yellow perch	0	0	0	1	0	0	−
Rana	Frog	3	0	0	6	0	6	−

(b) Forms from salt water.

Species tested	Chemical method						Yeast method						
	Muscle			Viscera			Muscle			Viscera			
	I	II	III	I	II	III	I	II	III	I	II	III	±
Homarus americanus Lobster	0	2	0	17	20	19	1	3	4	19	13	26	±
Littorina litorea Periwinkle	0	0	0	–	–	–	1	2	0	–	–	–	–
Ostrea edulis Oyster	1	0	4	–	–	–	0	0	5	–	–	–	–
Mya arenaria Clam	17	28	14	–	–	–	32	26	27	–	–	–	+
Mytilus edulis Mussel	29	27	39	–	–	–	45	36	35	–	–	–	+
Placopecten grandis Scallop	38	20	32	–	–	–	36	21	37	–	–	–	+
Asterias vulgaris Starfish	0	0	0	–	–	–	1	0	4	–	–	–	–
Squalus acanthias Dogfish	2	1	0	0	0	2	7	0	0	1	7	0	–
Raja senta Skate	0	1	4	1	1	0	0	0	2	2	2	4	–
Clupea harengus Herring	12	8	13	6	9	23	17	9	4	14	17	23	+
Pollachius virens Pollock	0	0	0	1	0	2	4	0	0	0	6	0	–
Gadus callarias Cod	0	1	5	0	0	4	3	0	0	4	2	0	–
Melanogrammus aeglefinus Haddock	2	1	0	0	1	4	0	1	0	1	0	0	–
Urophycis chuss Hake	0	4	1	0	3	5	0	0	0	0	3	0	–
Brosme brosme Cusk	5	0	3	6	4	0	3	5	0	0	0	1	–
Merluccius bilineatus Silver hake	1	6	7	0	0	1	3	2	0	0	0	0	–
Hippoglossus hippoglossus Halibut	0	0	2	4	0	0	1	5	1	0	7	1	–
Hippoglossoides platessoides Can. plaice	6	5	1	0	3	0	2	0	1	0	0	4	–
Glyptocephalus cynoglossus Witch	0	2	0	4	0	0	5	0	0	1	4	0	–
Pseudopleuronectes americanus Winter flounder	4	0	1	0	0	0	0	1	4	0	0	0	–
Limanda ferruginea Yellow tail	0	4	0	1	0	0	1	6	2	4	0	0	–
Scomber scombrus Mackerel	3	0	3	1	1	0	0	0	1	3	0	6	–
Myxocephalus octodecimspinosus Sculpin	0	1	0	0	0	0	3	5	0	0	0	6	–
Hemitripterus americanus Sea raven	0	4	0	0	1	2	4	0	4	2	4	1	–
*Cyclopterus lumpus** Lumpfish	3	3	3	2	0	1	0	1	2	0	0	2	–
Anarhichas lupus Catfish	2	4	0	0	1	0	1	2	0	3	0	2	–
Zoarces anguillaris Eelpout	2	0	0	1	3	1	4	4	1	5	0	0	–
Lophius piscatorius Goosefish	0	0	1	0	0	0	3	0	2	4	1	7	–

*Three analyses of one specimen.

The chemical method gave an error of not more than 8%, while the yeast fermentation method gave a result within 5% of the true vitamin content of pure solutions of thiamine hydrochloride. The spontaneous disappearance of the thiamine in the heated samples ranged from 20 to 30%. The per cent destruction due to thiaminase (table I) was calculated by subtracting the

thiamine values of the unheated samples from that of the heated samples. The losses found in the case of the smelt (*Osmerus mordax*) were much smaller than for some other species. Similarly Melnick *et al.* (1945) have found that herring (*Clupea harengus*) does not have as much thiaminase activity as different varieties of clams (*Mya arenaria*).

Evidence that the destruction was not caused by bacterial growth was obtained by making Breed (1928) smears at the end of the incubation period from several of the reaction mixtures containing actively destroying tissues. It was found that bacteria were not present in sufficient numbers to permit a count being made by this method and it is therefore extremely unlikely that bacteria were responsible for the thiamine losses.

Effect of Stomach Contents

In the analysis of the visceral organs of both white perch (*Morone americana*) and yellow perch (*Perca flavescens*) it was found that occasionally considerable destruction occurred in the unheated brei, if the stomach contents were not first thoroughly removed. The muscle preparations from these species were consistently without thiaminase. From the stomach of a yellow perch weighing 26.2 g. was removed a partially digested fish, (*Fundulus diaphanus*), weighing 3.6 g. The latter was tested for thiaminase activity and per gram, found to destroy 36 μg. of thiamine per hour (table II).

Table II. Thiaminase activity of the stomach contents of some white and yellow perch, as shown by per cent of thiamine destroyed in unheated samples, using the chemical method

No. of individual tested	Viscera intact			Viscera removed		
	1	2	3	4	5	6
Perca flavescens.....Yellow perch.....	29	18	18	0	1	3
Morone americana.....White perch.....	14	22	6	2	0	0

Residual Fermentation after Sulphite Treatment

It has been stated by Schultz, Atkin and Frey (1942) that sulphite treatment of samples for thiamine assay should give a measure of the fermentation activity of pyrimidines other than that held in the thiamine molecule, by virtue of the fact that such treatment inactivates specifically the thiamine fraction of the

Table III. Amounts of residual ferementation (per cent of fermentation remaining) after sulphite treatment using tissues with and without thiaminase.

No. of individual tested	1	2	3
Scallop.....(with thiaminase).....	13	21	26
Oyster.....(without thiaminase).....	4	4	6

sample. According to Deutsch (1944), however, thiamine is not so inactivated since he has found that a product of its treatment by sulphite, namely 4-amino-2-methyl-5-methyl sulphonic acid pyrimidine, has some fermentation activity. In table III is compared the data from the yeast fermentation tests in the case of scallop (*Placopecten grandis*) and oyster (*Ostrea edulis*) muscle.

DISCUSSION

Of the tissues examined in this survey the greater percentage containing thiaminase were found to be, in agreement with Deutsch and Hasler (1943), of freshwater origin. However, the enzyme is not by any means absent from certain truly marine forms. The *in vivo* function of this enzyme remains unsolved, but it is tempting to suggest that in the living organism it operates reversibly to synthesize thiamine. A somewhat less attractive theory would be that the enzyme could function to control the activity of co-carboxylase.

The considerable thiaminase activity of the stomachs of various species would be restricted to those individuals which feed on the smaller thiaminase-containing forms. The survey by Deutsch and Hasler (1943) has shown that many of the smaller freshwater fish contain the enzyme.

Sulphite treatment of the preparations containing thiaminase showed definitely higher fermentation activity than those from the heated preparations. It would seem, therefore, that in some cases the enzyme acted on the vitamin molecule to produce a substance which was resistant to destruction with sulphite but which still retained the fermentation activity. Thus Krampitz and Wooley (1944) observed that the vitamin activity of carp tissue thiamine mixtures was equal to that of the pure thiamine when tested with *Endomyces vernalis*, a yeast requiring only thiamine or its pyrimidine portion for growth (Wooley 1941) From the reaction mixture, 2-methyl-4-amino-5 hydroxymethylpyrimidine was. isolated and characterized.

SUMMARY

The viscera and muscle of a number of freshwater and marine animals have been tested for thiaminase activity. The enzyme was found in a greater proportion of the freshwater fish examined. In marine forms it was found only in one teleost and in four invertebrates. The stomach contents of some white and yellow perch contained the enzyme and this was shown to be caused by the fact that thiaminase happened to be present in the food of these individuals. Some evidence was found that the hydrolytic products of the thiamine molecule stimulate fermentation with the strain of yeast used in the vitamin assay.

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